Glycoprotein 60 Diversity in *Cryptosporidium parvum* Causing Human and Cattle Cryptosporidiosis in the Rural Region of Northern Tunisia

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Abstract. The zoonotic potential of *Cryptosporidium parvum* was studied in an extensive cattle farming region of northern Tunisia. Seventy fecal samples from pre-weaning calves and 403 fecal samples from children were examined by microscopy after modified Ziehl–Neelsen (MZN) staining. Positive *Cryptosporidium* specimens were identified at a species level using an 18S rRNA nested polymerase chain reaction (PCR) followed by an Restriction Fragment Length Polymorphism (RFLP) analysis. *C. parvum* isolates were subgenotyped by sequence analysis of the glycoprotein 60 (gp60) gene. Among calf samples, 14 samples were positive by MZN method. *C. parvum* was identified in all cases. Twelve *parvum* isolates (85.7%) belonged to family subtype IIa. Subtype IIaA15G2R1 was more prevalent (50%). Two *C. parvum* isolates corresponded to the IIdA16G1 subtype. Seven human samples were positive by MZN method. *C. parvum* and *C. meleagridis* were identified in four and three cases, respectively. Intraspecific characterization of *C. parvum* identified two subtypes, the IIaA15G2R1 and the IIdA16G1, also found in calves.

INTRODUCTION

The protozoan *Cryptosporidium* is a major public and animal health concern.[1] Immunocompromised people, young children, and pre-weaning animals are especially vulnerable. Until now, there is no affective treatment or vaccine commercially available to prevent the disease. Currently, 26 *Cryptosporidium* species have been named, and there is good evidence for 6 species as important causes of human cryptosporidiosis: *C. hominis*, *C. parvum*, *C. meleagridis* and occasionally, *C. cuniculus*, *C. felis*, and *C. canis*.[2] Despite occasional reports in livestock, *C. hominis* seems to be anthroponotically transmitted.[3] The other species are zoonotic and mainly transmitted from animals to human.[4] In animals, *C. parvum* and *C. meleagridis* are the most clinically and economically important gastrointestinal species in pre-weaning ruminants and birds, respectively.[2,3] In dairy cattle, *C. parvum* is mostly found in pre-weaning calves, whereas three other species, including *C. andersoni*, *C. bovis*, and *C. ryanae*, are found in older age groups.[3] In Tunisia, *Cryptosporidium* spp. was identified as a prevalent parasite in human and farm animals.[5,6] *C. hominis* and *C. parvum* were the dominant species in urban residents, whereas *C. parvum* and *C. meleagridis* were the causative species in children from rural areas.[4] *C. bovis* was found in lambs, and *C. meleagridis* was found in one broiler chicken.[6] Until now, no data are available about identification of subtypes of *Cryptosporidium* species.

Molecular characterization is essential in distinguishing human from non-human sources, understanding transmission, and strengthening the epidemiological evidence for causative links in outbreaks.[2] To characterize the transmission dynamics and zoonotic potential of *C. parvum*, numerous studies have been conducted to subtype *C. parvum* in humans and farm animals, especially calves.[3] One of the most popular subtyping tools is the DNA sequence analysis of 60 kDa glycoprotein (gp60). Sequence analysis of gp60 gene is widely used in *Cryptosporidium* subtyping because of its sequence heterogeneity and relevance to parasite biology. It is the single most polymorphic marker identified so far in the *Cryptosporidium* genome.[7,8] The gp60 gene is similar to a microsatellite sequence, having tandem repeats of the serine-coding trinucleotide TCA, TCG, or TCT at the 5′ end of the gene, and it presents extensive sequence differences in the non-repeat regions, which categorize *C. parvum* to several subtype families.[3] Among *C. parvum*, subtype families IIa and IId are found in both humans and ruminants to be responsible for zoonotic cryptosporidiosis. The IIc subtype family has, so far, been only found in humans.[3] The present study aims to improve the understanding of the epidemiology of *Cryptosporidium* in Tunisia by studying the genetic diversity of *C. parvum* in both populations of pre-weaning calves and humans from the same area.

MATERIALS AND METHODS

Fecal specimens and sample sites. Cattle breeding in Tunisia is observed mainly in the northern areas of the country, where bovine farming is supported by favorable climatic conditions. Livestock statistics for 2006 show that 65% of the cattle are in the north and that more than 40% of the pure breed cattle population is found in the two northern districts of Beja and Bizerte.[9] The study was performed in Bizerte District, which is located in northeast Tunisia (Figure 1). From April to October of 2007, 70 stool samples were collected immediately after defecation from calves less than 5 months in farms and private breeding units from the Joumine region (Figure 1). During the same period, 403 stool specimens were collected from children under 5 years of age; 258 stools (including 52 diarrheic specimens) were sampled from the pediatric rural communities (mean age = 33 months, SD = 17) living around farms animals of the Joumine region by a door to door survey, whereas 145 stools (including 51 diarrheic specimens) were collected in the healthcare unit of Menzel Bourguiba (mean age = 33 months, SD = 15), which is an important human settlement of Bizerte District (Figure 1).

Oocyst detection and species identification. Fresh stool specimens were examined for *Cryptosporidium* spp. oocysts.

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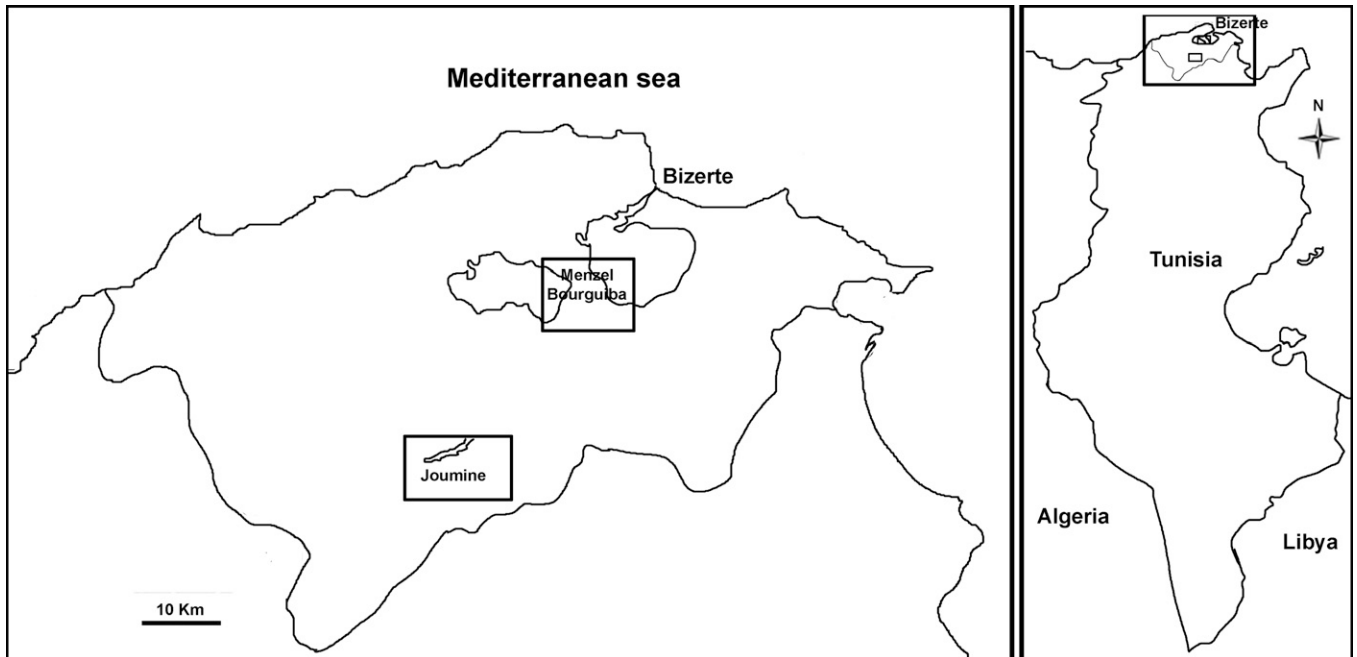†These authors contributed equally.

FIGURE 1.   Geographical situation of sample collection sites. The map of Tunisia on the right shows the geographical situation of the study district Bizerte (square). The photo on the left gives details of the geographical locations of the different sample collection sites Joumine and Menzel Bourguiba regions (squares).

Microscopic examination was carried out on smears of fecal concentrates (simplified version of Ritchie's formalin-ether sedimentation method)[10] after staining with the modified Ziehl–Neelsen technique (MZN).[11,12] DNA was extracted from all positive specimens using the QIAmp DNA Stool Mini-Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's recommendations. *Cryptosporidium* species were identified using a two-step 18S rRNA nested polymerase chain reaction (PCR) followed by an RFLP analysis as described by Coupé and others[5,13] with some modifications.

Intraspecific characterization of *C. parvum* isolates. Subgenotyping of *C. parvum* isolates was performed using nested PCR to amplify a fragment of the gp60 gene as described elsewhere.[1,7] Briefly, two-step nested PCR was used. AL3531 (5′-ATAGTCTCCGCTGTATTC-3′) and AL3535 (5′-GGA AGGAACGATGTATCT-3′) primers were used for the first-round PCR and AL3532 (5′-TCCGCTGTATTCTCAGCC-3′) and AL3534 (5′-GCAGAACCAGCATC-3′) primers were used for the second-round PCR to amplify a 840-bp fragment. Extracted DNA (1 µL) was mixed with a solution containing 200 nmol each primer, 200 µM 2'-deoxynucleoside 5'-triphosphate, 1.5 mM MgCl$_2$, and 2.5 U HotStar Taq polymerase (Qiagen GmbH, Hilden, Allemagne), with a final volume of 50 µL. Cycling conditions were an initial denaturation at 94°C for 10 minutes followed by 35 cycles of a three-step program (94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 1 minute). The amplified DNA fragments were purified using the Wizard Genomic DNA Purification Kit (Promega, Charbonnières, France) and sequenced in both directions with a Big Dye Terminator Cycle Sequencing Kit on an ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA). For each isolate, the *C. parvum* family group was assigned by sequence comparison with those isolates available in the GenBank database and published in

peer-reviewed international scientific journals (www.ncbi .nlm.nih.gov/blast). Subtypes were named on the basis of the number of TCA (A), TCG (G), and ACATCA (R) as described by Sulaiman and others.[14] and Gatei and others.[7] Nucleotide sequences were aligned with reference genotypes from GenBank using ClustalW and analyzed using Mega5 software.

The phylogenetic tree was created using the unweighted pair group method with arithmetic mean (UPGMA) based on evolutionary distances calculated by the Kimura two parameters. The reliability of branches was assessed by bootstrap analysis using 1,000 replicates. The topology of the *C. parvum* tree was constructed using *C. meleagridis* as outgroup.

RESULTS

Among calf samples, 15 (21.4%) samples were positive by the MZN method. PCR confirmed positivity, and RFLP analysis yielded typical restriction patterns for *C. parvum* in all cases. Intraspecific characterization of *C. parvum* isolates identified only two subtype families: IIa and IId (Figure 2); 13 of 15 *C. parvum* isolates (86.7%) belonged to family subtype IIa. The IIaA15G2R1 was the more prevalent (46.2%) subtype within this family (Table 1). The two latter *C. parvum* isolates corresponded to the IIdA16G1 subtype (Table 1).

Among human samples, seven (1.7%) samples were positive by the MZN method. PCR confirmed positivity, and RFLP analysis yielded typical restriction patterns for *C. parvum* in four cases and *C. meleagridis* in three cases. Intraspecific characterization of *C. parvum* isolates identified only two subtypes: IIaA15G2R1 in two cases and IIdA16G1 in two other cases (Table 1). *Cryptosporidium* species and *C. parvum*
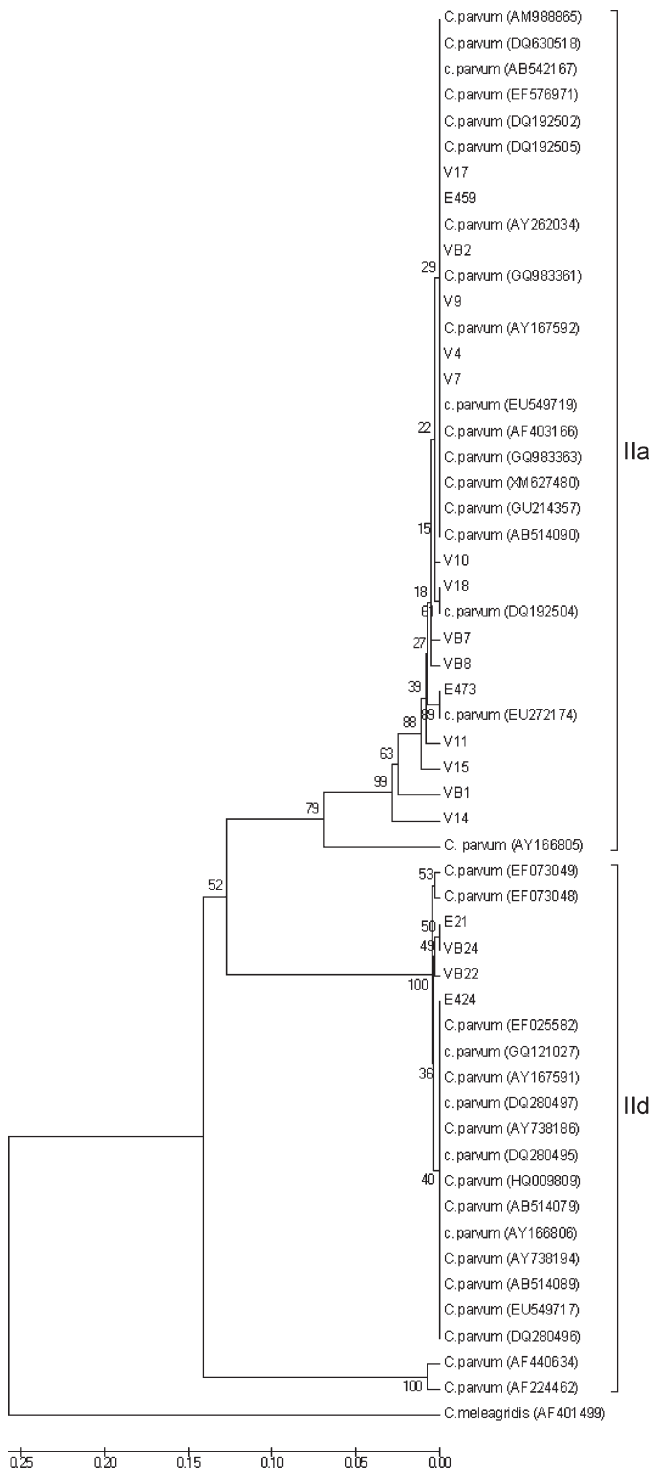
FIGURE 2. Phylogenic analysis of the gp60 nucleotide sequences of *C. parvum* strains from humans and calves (tree obtained by the method of UPGMA with *C. meleagridis* as an outgroup and bootstrap values based on 1,000 replicates). Our samples: E459, E473, E21, and E424 were from humans and VB2, V7, V9, V10, V4, V17, V18, VB7, VB8, V11, V15, VB1, V14, VB22, and VB24 were from calves. Other nucleotide sequences were from GenBank.

TABLE 1
Cryptosporidium species and *C. parvum* subtypes identified in human population according to epidemiological data

Study area and code	Age group (years)	Sex	Stool consistency	*Cryptosporidium* species	*C. parvum* subtype family	*C. parvum* subtypes
Joumine region						
E21	3–4	Female	F	*C. parvum*	IId	A16G1
E424	3–4	Male	D	*C. parvum*	IId	A16G1
E183	3–4	Male	F	*C. meleagridis*		
E204	4–5	Female	F	*C. meleagridis*		
Menzel Bourguiba region						
E459	2–3	Male	D	*C. parvum*	IIa	A15G2R1
E473	2–3	Male	D	*C. parvum*	IIa	A15G2R1
E250	2–3	Male	F	*C. meleagridis*		

D = diarrheic; F = formed.

subtypes according to epidemiological data are reported in Table 2.

Phylogenic analysis of all *C. parvum* strains was shown in Figure 2.

DISCUSSION

Conventional procedures for oocyst concentration and detection in stool specimens, namely formalin-ether sedimentation and acid-fast staining, were used for *Cryptosporidium* screening. However, the high threshold necessary for oocyst detection by these coprodiagnosis methods, particularly in formed or semiformed stool specimens, could limit oocyst detection in asymptomatic individuals and underestimate the number of positive samples.[15,16]

Prevalence rate of *Cryptosporidium* spp. infection among children less than 5 years old was particularly low (1.7%). This rate may be partly because of the sample processing methodology but also could have been related to an absence of close contact between the human population sampled and animals. *C. meleagridis* was found almost as frequently as *C. parvum*. This finding suggests that zoonotic transmission from poultry is one of the most important causes of human cryptosporidiosis in rural communities of Tunisia, where most families have their own poultry breeding close to their houses. However, even in this farming region, families do not necessarily have their own cattle, which suggest a low direct contact with these animals. Nevertheless, *C. parvum* was the only specie identified in the positive calf stool specimens collected in the same region, which provides evidence of possible association of infected calves and human infection with *C. parvum*. The role of cattle in the zoonotic transmission of *C. parvum* in northern Tunisia was further supported by gp60 subgenotyping data.

TABLE 2
C. parvum subtypes identified in calves from Bizerte District

Subtype family and subtype	Number of isolates in humans	Number of isolates in calves
IIa		
A15G2R1	2	6
A16G2R1	0	3
A13G2R1	0	2
A20G3R1	0	1
IId		
A16G1	2	2

As reported by other studies, family subtype IIa was the dominant family in calves.[3] Also, the IIaA15G2R1 seemed to be the most common subtype on the dairy farms.[17,18] Interestingly, two of four (50%) *C. parvum* isolates from children belonged to family subtype IIa and were similar to the IIaA15G2R1 subtype (the most prevalent subtype identified in calves during the same period), which suggests that family subtype IIa and particularly, IIaA15G2R1 subtype can spread easily within cattle populations and be transmitted to humans as well. In fact, this variant is frequently observed in *C. parvum* populations worldwide and has been widely reported in zoonotic infections.[1] As a risk factor for human cryptosporidiosis, contact with cattle was implicated in the neighboring countries, such as Egypt and Spain,[4,19] as well as the United States, United Kingdom, Ireland, and Australia.[20–23] However, in our study, taking into account the age of children and their kind of life (human settlement), contact with livestock is possible; however, drinking raw (unpasteurized) milk may also represent a probable risk factor for *Cryptosporidium* transmission in this area.[24–26] More investigations should be performed with large and more representative samples.

As reported in European countries, the IId subtype family was occasionally found in calves in addition to IIa subtypes.[3] In this study, the IIdA16G1 was identified in two calves as well as two children living in the rural environment around farm animals. Interestingly, IId subtypes of *C. parvum* have never been found in humans in the United States and Canada, where they are absent in calves.[3] Thus, the less common bovine *C. parvum* subtype family IId may potentially also be responsible for some zoonotic infections in northern Tunisia. However, the relatively high proportion of cases (50%) in humans highlights the need for additional epidemiological investigations. In fact, in regions where both subtypes IIa and IId are found (i.e., Spain), family subtype IIa infects preferentially calves, whereas family subtype IId has a tropism for lambs and kids.[19] Although an earlier study conducted in Tunisia suggested that *C. parvum* was absent in sheep,[22] studies involving more animal samples from lambs are needed for a better understanding of the sources of *Cryptosporidium* human infections in this northern area.

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